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14. ABSTRACT Bone is the frequent metastatic site for human breast cancer resulting in significant morbidity and mortality in patients with advanced disease. Osteoprotegerin (OPG) is a "decoy" receptor that competes with RANK for RANKL, thus, modulating the effects of RANKL. However, during the metastatic events involving cancer and stromal cell interaction, endogenous OPG levels are markedly reduced. Thus, OPG remains an effective molecule for future therapies for bone metastasis. We sought to achieve sustained effects of OPG combining cell therapy and gene therapy approaches. The aims were to determine therapeutic effects of stable OPG expression by rAAV gene therapy in a murine model of breast cancer bone metastasis, and to determine the synergistic effects of OPG gene therapy with bisphosphonate therapy in a murine model of breast cancer bone metastasis. So far, we produced high-titer recombinant AAV vectors encoding osteoprotegerin, and tested the feasibility of MSC therapy for reducing osteolysis in bone initiated by cancer growth. Also we established a method for bone homing of ex vivo cultured MSC by transient expression of $\alpha 4 \beta 1$ integrin. Continuation of the ongoing studies in to next year will provide valuable information on therapeutic effects of this therapy for breast cancer bone metastasis.					
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INTRODUCTION

Bone is the frequent metastatic site for human breast cancer resulting in significant morbidity and mortality in patients with advanced disease. A vicious cycle, arising due to the interaction of cancer cells and the bone microenvironment results in the upregulation of factors promoting osteoclastogenesis and osteolytic bone destruction. Thus, osteolysis and tumor cell accumulation can be inhibited by interrupting one or more of the steps involved in the cycle. The major treatment to reduce the burden of bone metastasis in breast cancer patients is bisphosphonate therapy. Despite significant efforts to improve the potency of bisphosphonates, the complications are only retarded but not prevented. Thus, while improving the formulations of bisphosphonate compounds, development of newer therapies that can both ameliorate the threshold of bone destruction and increase survival of patients with metastatic breast disease will be highly beneficial.

A better understanding of the molecular events in breast cancer osteolytic bone destruction indicates that the receptor activator of nuclear factor κ B ligand (RANKL), produced by osteoblasts, activated T cells and marrow stromal cells stimulates the recruitment, differentiation, and activation of osteoclasts by binding to RANK. Osteoprotegerin (OPG) is a “decoy” receptor that competes with RANK for RANKL, thus, modulating the effects of RANKL. However, during the metastatic events involving cancer and stromal cell interaction, endogenous OPG levels are markedly reduced. Thus, OPG remains an effective molecule for future therapies for bone metastasis. To achieve sustained effects of OPG, gene therapy is more powerful than pharmacological therapies. Since the process of bone metastasis in breast cancer is a secondary event that occurs in late-stage disease or during recurrence, genetic therapies aimed at controlling this process should be both sustained and localized. Thus, for sustained expression of therapeutic levels of OPG, a vector capable of stable expression of the transgene without vector-associated toxicity and immunity is ideal. The adeno-associated virus vectors (AAV) are more promising to this end. With recombinant AAV vectors, it is possible to obtain significant therapeutic advantage by either systemic or bone-targeted transduction and can be combined with bisphosphonate treatment for synergistic effects.

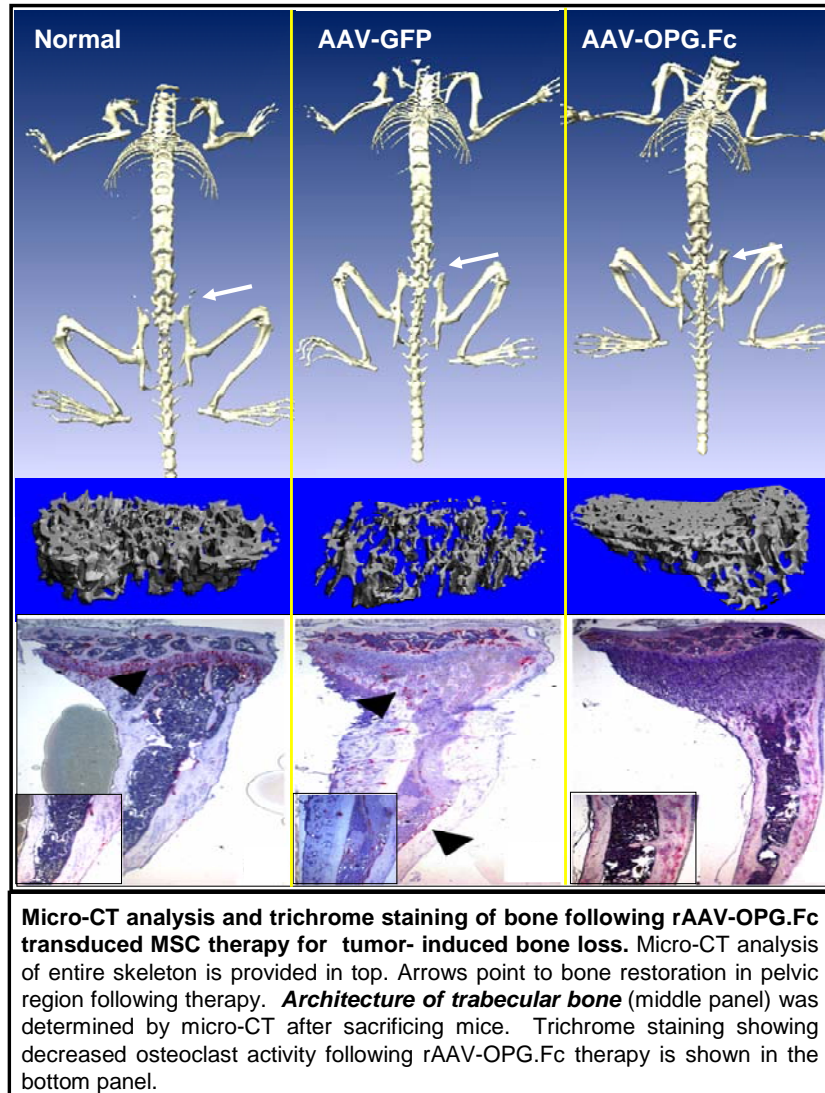
The proposed specific aims of the project are:

- 1) To determine the therapeutic effects of stable OPG expression by rAAV gene therapy in a murine model of breast cancer bone metastasis, *and*
- 2) To determine the synergistic effects of OPG gene therapy with bisphosphonate therapy in a murine model of breast cancer bone metastasis.

BODY

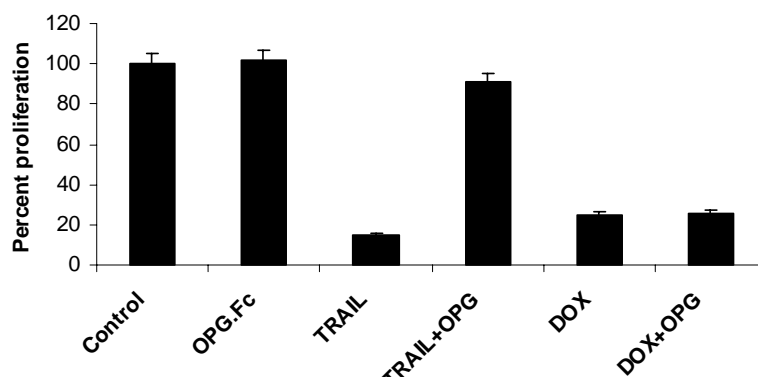
Recombinant AAV-mediated, systemically stable expression of osteoprotegerin inhibits osteolytic bone damage. The purpose of the present study was to determine the effect of sustained level of OPG.Fc in a therapeutic model of osteolytic breast cancer. 6 weeks old female athymic nude mice (n=24) were injected intra-cardiac 2×10^5 MDA-MB-435 breast cancer cells expressing firefly luciferase. 3 mice served as normal age matched control and received no tumor cells or treatment. Widespread skeletal metastases were confirmed 7 days after the intra-cardiac injection after bioluminescence imaging. 16 out of 24 mice showed clear signs of bone metastasis. At this point 8 out of 16 positive mice were administered intra-muscular delivery of 3×10^{11} virus particles of AAV-OPG.Fc and rest of the mice received 3×10^{11} particles of AAV-GFP. Therapeutic benefits were determined 4 weeks after the delivery of the virus particles. Bioluminescence imaging showed significant reduction in tumor growth in the OPG.Fc treated mice compared to GFP treated mice. No significant difference was observed in bioluminescence from any extra-osseous metastasis between OPG.Fc and GFP treated mice. Micro-computerized tomography (μ CT) analysis indicated significant decline in bone to tissue volume ratio and trabecular bone density in the tumor challenged untreated or GFP treated animals. OPG.Fc treatment restored the bone to tissue volume ratio and significantly enhanced the trabecular bone density. Ki-67 immunostaining showed presence of proliferating tumor cells in the tibia of both OPG.Fc treated and GFP treated animals. PARP-p85

immunostaining was detected only in the tumor cells which are trapped inside the trabeculae of the newly formed cancellous or trabecular bone in the metaphyses and not in the tumor cells those were present in the diaphyses suggesting indirect killing of tumor cells by OPG.Fc. A significant loss in body weight was observed in both OPG.Fc treated and GFP treated mice which suggest inability of OPG.Fc to influence any soft tissue metastasis. Moreover, OPG.Fc treatment resulted into overproduction of bone, which may require introduction of regulated gene expression system. We suggest that OPG.Fc in combination with chemotherapy may prove useful for the management of osteolytic bone metastasis.

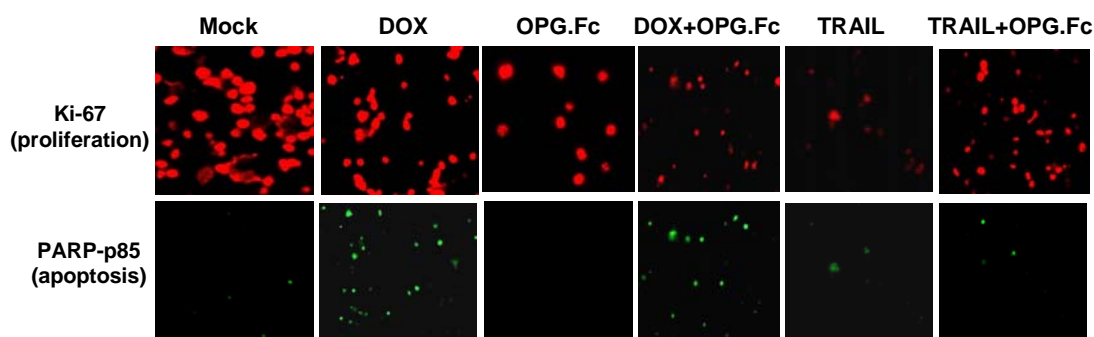


Doxorubicin induces cancer cell apoptosis independently of TRAIL blocking by OPG. Since TRAIL binding property of OPG inhibits apoptosis of metastasized cancer cells in the bone marrow, we reasoned that combination of OPG therapy with chemotherapy using drugs capable of inducing cancer cell apoptosis, independently of TRAIL pathway, would provide great synergy. To determine this, we used doxorubicin, a known chemotherapeutic drug commonly given to patients with breast cancer. Human osteolytic cancer cell line, CAG, was seeded at a concentration of 2×10^5 cells/well of a 6-well dish and treated with 50 ng/ml TRAIL, 1 μ g/ml purified OPG.Fc and 20 ng/ml doxorubicin in various combinations. The cells were harvested after 48 hours, washed in PBS and subjected to Trypan Blue staining for cell viability. Cell proliferation was determined by MTT assay and immunostaining performed in a cytospin using cell proliferation marker Ki-67 and apoptosis marker PARP-p85 fragment. Results of these studies, shown in figures below demonstrate that although in the presence of OPG, TRAIL-mediated apoptosis is significantly prevented, apoptotic effects of DOX was totally

unaffected in the presence of OPG. Thus, combination of DOX with bone-targeted OPG therapy is expected to prevent osteolytic bone damage and promote tumor cell apoptosis to increase survival.



Tumoricidal effects of doxorubicin is independent of TRAIL binding of OPG. Human osteolytic cancer cell line, CAG, were seeded at a concentration of 2×10^5 cells/well of a 6 well dish and treated with 50ng/ml TRAIL, 1 μ g/ml mouse OPG.Fc and 20ng/ml doxorubicin (DOX) in various combinations in duplicates. MTT assay for cell proliferation was performed 48 hrs later.



Proliferation and apoptosis of human osteolytic cancer cell line following treatments with OPG, DOX and TRAIL combinations. Human osteolytic cell line CAG was seeded at a concentration of 2×10^5 cells/well of a 6 well dish and treated with 50ng/ml TRAIL, 1 μ g/ml mouse OPG.Fc and 20ng/ml doxorubicin in various combinations in duplicates. Cells were harvested after 48 hours, centrifuged to pellet the cells, washed in PBS and analyzed by immunostaining for cell proliferation marker (Ki-67-Alexa fluor-594, red) and apoptosis marker (PARP-p85 fragment, Alexa Fluor-488, green).

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that systemically stable expression of OPG using rAAV is capable of decreasing osteolytic bone damage in a mouse model of metastatic breast cancer.
- Established that doxorubicin acts independently of the influence of OPG on TRAIL binding.

REPORTABLE OUTCOMES

(Papers published or communicated)

Isayeva, I., Chanda, D., Eltoum, I-E., and Ponnazhagan, S. Effects of sustained anti-angiogenic therapy in multi-stage prostate cancer in TRAMP mice. Cancer Res. 2007, 67:5789-5797.

Chanda, D., Isayeva, T., Kumar, S., Szafran, A.A., Zinn, K., Ponnazhagan, S. Systemic osteoprotegerin gene therapy restores tumor-induced bone loss in a therapeutic model of breast cancer bone metastasis. Cancer Res. 2007 (in communication).

(Results presented in conferences)

Chanda, D., Isayeva, T., Kumar, S., Szafran, A.A., Zinn, K., Ponnazhagan, S. Systemic osteoprotegerin gene therapy restores tumor-induced bone loss in a therapeutic model of breast cancer bone metastasis. 10th Annual Meeting of the American Society for Gene Therapy, Seattle, WA, June 2007.

CONCLUSIONS

We have successfully determined that sustained expression of OPG using recombinant AAV greatly reduces osteolytic bone damage in a mouse model of bone metastatic breast cancer. We will continue to determine if the same therapy using mesenchymal stem cell vehicle would provide better benefits. In addition we will combine the use of chemotherapy, targeted to the cancer cells in addition to inhibiting osteoclast activity by OPG. Continuation of the ongoing studies in to next year will provide valuable information on therapeutic effects of this therapy for breast cancer bone metastasis.

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Selvarangan Ponnazhagan, Ph.D.

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REFERENCES

N/A

APPENDICES

N/A